REMARKS

This Amendment After Final is submitted in reply to the Office Action mailed on April 4, 2006. In the Office Action, the Examiner rejected claims 13-20, 22-28, 30, 42-62, and 64-87. With this Amendment After Final, claims 13-20, 22-28, 30, 42-62, and 64-87 are canceled, and new claims 88-122 are added. Claims 1-12, 21, 29, 31-41, and 63 were previously cancelled. Upon entry of this Amendment After Final, the above-identified application will include claims 88-122.

Though claims 13-20, 22-28, 30, 42-62, and 64-87 are canceled via this Amendment After Final, Applicants continue to believe claims 13-20, 22-28, 30, 42-62, and 64-87 are allowable, as originally presented in the above-identified application and also as claims 13-20, 22-28, 30, 42-62, and 64-87 presently exist as of the present request to cancel claims 13-20, 22-28, 30, 42-62, and 64-87. Therefore, Applicants are canceling claims 13-20, 22-28, 30, 42-62, and 64-87 without prejudice to Applicants' right to pursue claims worded like claims 13-20, 22-28, 30, 42-62, and 64-87, as originally presented or as worded subsequent to original presentation, in the above-identified application or in any continuing application that is based on the above-identified application. Furthermore, no claim cancellation made herein is related to any statutory patentability requirement unless expressly stated herein.

Examiner's Objection to the Claims

In the Office Action, the Examiner objected to claims 17 and 18 under 37 C.F.R. §1.75(c) as allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. According to the Examiner:

> The instant claims depend from base claims which have two requirements: 1) the DNA molecule must encode a porcine adipocyte leptin and 2) must hybridize to a specified sequence. The dependent claims 17-18 place size limitations on the DNA of "at least 20" or "at least 50" bases, which is no where near the necessary size of a DNA which will encode a porcine leptin polypeptide, absent evidence to the contrary. Therefore, the claims do not appear to further limit the claims from which they depend.

> Claims 17 and 18 appear to be broader than the claim from which it depends. Claim 13 is directed to a DNA molecule that encodes a porcine leptin, wherein the DNA hybridizes to a specified molecule with a specified sequence. Claim 17 appears to encompass any fragment ("at least about 20 bases and encodes at least a fragment") and claim 18 appears to encompass any fragment ("at least about 50 bases and encodes at least a fragment"). This language encompasses many more types of molecules than does claim 13, based on the fragment language, and therefore, is broader in nature. Because it is broader, it does not further limit the base claim.
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> It is additionally noted that stringent hybridization conditions for different length nucleic acid molecules varies, therefore, it is not clear if the conditions listed in claim 13 would be the same or different from those in claims 17 and 18, which also makes the claims indefinite

Claims 17-18, as of issuance of the present Office Action, read as follows:

> *17. (Currently Amended) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule is at least about 20 bases and encodes at least a fragment of the porcine leptin polypeptide that hybridizes to the nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.*
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> *18. (Currently Amended) The isolated single or double-stranded DNA molecule of claim 13 wherein the isolated DNA molecule is at least about 50 bases and encodes at least a fragment of the porcine leptin polypeptide that hybridizes to the nucleotide sequence of SEQ ID NO: 1 under stringent hybridization conditions.*

Applicants disagree with the Examiner's contention regarding molecule lengths needed to encode a DNA molecule and note the Examiner has not advanced any evidentiary support for the Examiner's contention. Nonetheless, Applicants have cancelled claims 17 and 18, as indicated above, for reasons unrelated to the Examiner's objection to claims 17-18. Consequently, the Examiner's objection to claims 17-18 is moot.

Applicants also note the Examiner made a comment about alleged indefiniteness of claims 17-18 while reciting the present objection to claims 17-18. Applicants have not addressed this comment about alleged indefiniteness, since this comment did not pertain or support the Examiner's stated objection to claims 17-18.

Claim Rejections Under the Enablement Requirement of the First Paragraph of 35 U.S.C. §112

In the Office Action, the Examiner rejected claims 13-15, 17-20, 25, 30, 41-42, 44, 50, 52, 53, 55, 57, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80-82 under 35 U.S.C. §112, first paragraph, as allegedly failing to satisfy the enablement requirement:

> The first step in determining if a claim meets the enablement requirements of 35 U.S.C. 112, first paragraph, is understanding what is being claimed. The instant claims are directed to nucleic acid molecules which encode a porcine leptin polypeptide, wherein the nucleic acid hybridizes to at least about 20-50 bases of SEQ ID NO:1, 20-50 bases of SEQ ID NO:3, or wherein the nucleic acid molecule is at least 20-50 bases long. It is clear that the instant specification encompasses and intends for fragments of porcine leptin to be encompassed in the scope of the invention. However, the instant specification only describes a single protein which can be called "porcine adipocyte polypeptide leptin" or "porcine leptin polypeptide" or "porcine leptin polypeptide leptin", and this protein is 166 amino acids in length with the signal sequence and 145 amino acids in length without the signal sequence. The prior art nucleic acid molecules which encode leptin are also described in Figure 4, which encode a leptin of a similar length to that of the disclosed porcine leptin. The specification distinguishes fragments from the "leptin" depicted in Figure 2 at page 7 of the specification; "[a]lso intended within the scope of the present invention is any polypeptide having at least about 8 amino acids present in the above-mentioned sequence." Therefore, the claims are directed to nucleic acid molecules which encode porcine leptin (functional limitation) wherein the nucleic acid molecule hybridizes to at least about 20 (or 50) nucleotides of a disclosed nucleic acid molecule or wherein the isolated nucleic acid molecule is at least about 20 (or 50) bases in length (structural limitation).
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> First, the art does not recognize a nucleic acid as short as 20-50 nucleotides long that encodes a leptin molecule and the instant specification fails to teach a molecule meeting this limitation. The specification does teach that a fragment of 20 nucleotides is intended in the scope of the claims, but it does not teach that this length is sufficient for encoding leptin as defined in the instant specification as corresponding to SEQ ID NO:2. Therefore, one of ordinary skill in the art would not find such a length sufficient for encoding a leptin molecule from pigs, absent evidence to the contrary, and the claims are not enabled for such. Next, SEQ ID NO:1 is a genomic sequence with significantly long stretches of non-coding regions. The claims encompass isolated DNA which hybridizes to at least 20 or 50 nucleotides of SEQ ID NO:1, however, the vast majority of the nucleic acid molecules which hybridize (again, no conditions are provided, so the majority of

> nucleic acids in existence would hybridize under various conditions) to 20 or 50 bases would not meet the functional requirements of the claims, which are to encode a porcine leptin polypeptide. The structure which is given is not sufficient to result in the required function of the claims, and the claims are not enabled.
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> Applicant argues the rejection at pages 25-30 of the response. However, Applicant's arguments are based on the premise that a nucleic acid molecule of "at least about" 20 bases encodes porcine leptin. For the reasons given above and supported by the disclosure of the instant specification, this is a false premise. Therefore, the rejection is maintained for the reasons of record and for those reasons given above. Applicant may wish to amend the claims to eliminate the functional requirement that the isolated nucleic acid molecule encode porcine leptin, and this may obviate this ground of rejection.

Despite the Examiner's comments, claims 13-15, 17-20, 25, 30, 41-42, 44, 50, 52, 53, 55, 57, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80-82 that were in the present application when the present Office Action issued are enabled by the disclosure in accordance with the first paragraph of 35 U.S.C. §112. However, as noted above, Applicants have cancelled claims 13-15, 17-20, 25, 30, 41-42, 44, 50, 52, 53, 55, 57, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80-82 for reasons unrelated to the Examiner's enablement rejection of claims 13-15, 17-20, 25, 30, 41-42, 44, 50, 52, 53, 55, 57, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80-82. Consequently, the Examiner's enablement rejection of claims 13-15, 17-20, 25, 30, 41-42, 44, 50, 52, 53, 55, 57, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80-82 is moot.

Nonetheless, some clarifying comments about statements of the Examiner in support of the enablement rejection are provided herein. Once evidence of enablement for a claimed invention is provided, the burden of proof to establish the evidence is erroneous is the Examiner's burden to bear. In the Amendment filed in November, 2004 in reply to the June, 2004 Office Action, Applicants pointed out where evidence of enablement for the claimed invention existed in the above-identified application. The Examiner, in the present Office Action, does not provide any explanation as to why Applicant's proffered disclosure is erroneous, but instead merely alleges "it does not teach that this length is sufficient for encoding leptin as defined in the instant specification as corresponding to SEQ ID NO:2." This explanation is inadequate and does not shift the burden regarding enablement back to Applicants,

since the Examiner merely focuses on the length of sequences disclosed in the application and fails to address the evidence of enablement Applicants previously advanced.

Furthermore, the dispute that is the real basis of this enablement rejection continues to center on the meaning of the term "encode" and the ability of shorter nucleotides to encode porcine leptin. The Examiner continues to challenges Applicant's definition of encode as allegedly being erroneous. Applicant has provided evidence to the Examiner demonstrating support for Applicant's definition of encode. See the discussion on pages 27 and 28 of the Amendment After Final filed November 23, 2004 in response to the Final Office Action dated June 3, 2004 along with the article by Y.M. Kennes, B.D. Murphy, F. Pothier and M.-F Palin, entitled Characterization of Swine *Leptin (Lep)* Polymorphisms and Their Association with Production Traits (2001) attached as Exhibit A of the Amendment After Final filed November 23, 2004 in response to the Final Office Action dated June 3, 2004.

The Kennes article demonstrates, despite the Examiner's allegation to the contrary, that the scientific literature does indeed recognize nucleic acid molecules having at least about 20 bases of a nucleotides sequence derived from a leptin gene that encodes a leptin molecule. The Examiner continues to allege "the art does not recognize a nucleic acid as short as 20-50 nucleotides long that encodes a leptin molecule." However, despite the fact Applicant has provided evidence in support of Applicants' understanding of the meaning of "encode," the Examiner has not yet produced any such evidence in support of the Examiner's contention that Applicant's interpretation is wrong. The Examiner instead now merely points to the length of sequences disclosed in the application, but does not produce any evidence establishing that nucleic acid molecules having at least about 20 bases of a nucleotides sequence derived from a leptin gene cannot encode a leptin molecule. The Examiner's mere allegation about what the art does not recognize is insufficient to carry the Examiner's obligation under the enablement requirement.

Claim Rejections Under the Second Paragraph, 35 U.S.C. §112

In the Office Action, the Examiner alleged Applicants' use of "at least about" to characterize the number of bases (length) of a molecule in claims 17 and 18 and to characterize

the number of bases of a sequence to which a molecule hybridizes in claims 14, 15, 19, 20, 44, 52, 55, 57, 60, 62, 64, 66, 70, 74, 78, 80, 82 is indefinite. In support of this rejection, the Examiner alleged:

> Claims 14, 15, 17, 18, 19, 20, 44, 52, 55, 57, 60, 62, 64, 66, 70, 74, 78, 80, 82 are indefinite for the recitation "at least about" in conjunction with a number of nucleotides which are to hybridize. This recitation is indefinite because the lower limits of what are to be encompassed by the claims are not clear. The instant specification does not indicate what range "at least about" is meant to encompass. Furthermore, "at least" is in direct conflict with "about" since "at least" sets a lower limit to the range, but "about" changes that limit. Therefore, the claims are indefinite because the metes and bounds of "at least about" cannot be determined.
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> Applicant asserts that the term "at least about X could alternatively be written as "about X or more" and "no one of ordinary skill in the art would be confused about the meaning of "at least about X". Applicant's argument has been fully considered but is not found to be persuasive. The phrase "at least" has a definite meaning; it sets a very definite lower limit for the number of nucleotides which are to hybridize. The term "about" is not specific to the precise number of nucleotides which are to hybridize. The use of the two phrases/terms together makes the claims indefinite because the metes and bounds of the number of nucleotides which are to hybridize cannot be determined. For example, does the claim encompass 15 nucleotides? Would 25 nucleotides be encompassed by "at least about 50"? Does the claim encompass 10 nucleotides? The skilled artisan would have no idea if they were infringing the claim because the metes and bounds are not clear and definite. The rejection is maintained for the reasons of record.

Despite the Examiner's allegations, claims, 14, 15, 17, 18, 19, 20, 44, 52, 55, 57, 60, 62, 64, 66, 70, 74, 78, 80, 82 that were in the present application when the present Office Action issued are believed definite in accordance with the second paragraph of 35 U.S.C. §112. However, as noted above, Applicants have cancelled claims 14, 15, 17, 18, 19, 20, 44, 52, 55, 57, 60, 62, 64, 66, 70, 74, 78, 80, 82 for reasons unrelated to the Examiner's indefiniteness rejection of claims 14, 15, 17, 18, 19, 20, 44, 52, 55, 57, 60, 62, 64, 66, 70, 74, 78, 80, 82. Consequently, the Examiner's indefiniteness rejection of claims 14, 15, 17, 18, 19, 20, 44, 52, 55, 57, 60, 62, 64, 66, 70, 74, 78, 80, 82 is moot.

Nonetheless, some clarifying comments about statements of the Examiner in support of the indefiniteness rejection are provided herein. The Examiner discounts Applicants' explanation about how the term "at least about X" could alternatively be written as "about X or more" without explanation. The Examiner counters with questions about whether 10 or 25 nucleotides would be encompassed by the term "at least about 50." Applicants previously admitted the "about 20" and the "about 50" portions of these terms are limited to minor variations from the base number (20 or 50 under the present facts). The answer to the Examiner's question is whether 10, or even 25, amount to minor variations from 50. Though the terms "at least about 20" and "at least about 50" are not exact, the indefinite query doe not go to whether the subject terminology is exact. Applicants continue to see no basis for believing one of ordinary skill in the art would not reasonably be able to determine the scope of the terminology at issue.

Next, the Examiner stated that Applicants' use of the term "stringent hybridization conditions" in various claims allegedly renders claims 13-20, 22-28, 30, 42-62, 64-87 indefinite:

> Claims 13-20, 22-28, 30, 42-62, 64-87 are indefinite for the limitation of "stringent hybridization conditions". The limitation "stringent hybridization conditions" is equivalent to reciting a range without indicating the metes and bounds of the conditions since there is no indication of what conditions are to be encompassed by the claims. The specification does not provide a definition of what conditions are considered "stringent" and the art recognizes a multitude of conditions which could be used and considered "stringent". Because a multitude of conditions are encompassed by the claims, it is not clear which molecules which may hybridize under varying conditions are encompassed by the claims. Therefore, the metes and bounds of the claims are unclear and the claims are indefinite.
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> Applicant argues this rejection at pages 27-30. Applicant's arguments have been considered, but are not deemed to be persuasive. Applicant states that the use of broad terminology does not necessarily render a claim indefinite. Applicant is correct in saying that breadth does not equate to indefiniteness. However, this is not the case in the instant application. The metes and bounds of the claims cannot be determined because the claims encompass a wide host of molecules depending on which conditions are intended by the terminology "stringent hybridization

> conditions" and those skilled in the art would not know which conditions are intended by the claims because the metes and bounds of what is covered by the claims is unclear. In the absence of a true definition in the specification that indicates what conditions are intended by "stringent", the rejection is maintained for the reasons of record.

Despite the Examiner's allegations, Applicants do not believe use of the "stringent hybridization" terminology renders any of claims 13-20, 22-28, 30, 42-62, 64-87 that were in the present application when the present Office Action issued indefinite under the second paragraph of 35 U.S.C. §112. However, as noted above, Applicants have cancelled claims 13-20, 22-28, 30, 42-62, 64-87 for reasons unrelated to the Examiner's indefiniteness rejection of claims 13-20, 22-28, 30, 42-62, 64-87. Consequently, the Examiner's indefiniteness rejection of claims 13-20, 22-28, 30, 42-62, 64-87 is moot.

Nonetheless, some clarifying comments about statements of the Examiner in support of the indefiniteness rejection are provided herein. In support of the present rejection, the Examiner states "In the absence of a true definition in the specification that indicates what conditions are intended by 'stringent'", the rejection will be maintained. The issue is whether one of ordinary skill in the art would be able to reasonably understand the scope of the claim language, not whether there is an actual definition in the specification for all claim terminology. The Examiner's basis for alleging the stringent hybridization conditions terminology is indefinite is therefore clearly erroneous. A hard and fast definition is unnecessary if those of ordinary skill in the art would understand what is meant by stringent hybridization conditions. The Examiner has not produced any evidence establishing that one of ordinary skill in the art would be unable to reasonably understand the scope of the stringent hybridization condition claim language. Therefore, the Examiner has effectively done nothing more than question the definiteness of the stringent hybridization condition claim language. This does not, however, establish that the subject claim language is in fact indefinite.

Furthermore, Applicants note that nucleic acid hybridization, even as far back as 1989, was considered to be well understood:

Nucleic Acid Hybridization

> This is the most commonly used and reliable method of screening cDNA libraries for clones of interest. None of the other methods displays such an abundance of attractive features. Screening by nucleic acid hybridization allows extremely large numbers of clones to be analyzed simultaneously and rapidly, does not require that the cDNA clones be full-length, and does not require that an antigenically or biologically active product be synthesized in the host cell. **Furthermore, as a result of more than 20 years of work, the theoretical basis of nucleic acid hybridization is well-understood**. This has led to the development of a large number of different techniques that can accommodate nucleic acid probes of different lengths and specificities.

Sambrook, Joseph; Fritsch, Edward F.; Maniatis, Thomas; Molecular Cloning, A Laboratory Manual, Volume II, p. 8.46, Second Edition (1989) (Emphasis added; incorporated by reference in the priority application US 08/692,922); attached as Exhibit A of this Amendment After Final.

The invention defined in the claims (now cancelled) rejected by the Examiner in the present Office Action belongs to the porcine leptin nucleotide genus and hybridizes to the nucleotide sequence of SEQ ID NO: 1 under stringent conditions. The Examiner is reminded the patent specification is not required to teach every detail in the related art because the specification is directed toward one of skill in the art "and preferably omits, what is well known in the art." Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1384, 231 U.S.P.Q. (BNA) 81, 94 (Fed. Cir. 1986). Ira Donner in Patent Prosecution, Fourth Edition, Volume Two, p. 1515, states "[t]he Board of Patent Appeals and Interferences (Board) has further stated as follows":

> In rejecting a claim under the second paragraph of 35 USC 112, it is incumbent on the examiner to establish that one of ordinary skill in the pertinent art, when reading the claims in light of the supporting specification, would not have been able to ascertain with a reasonable degree of precision and particularity the particular area set out and circumscribed by the claims. Citing Ex Parte Wu, 10 USPQ2D 2031, 2033 (B.P.A.I. 1989) (citing In re Moore, 439 F.2d 1232, 169 USPQ 236 (C.C.P.A. 1971); In re Hammack, 427 F.2d 1378, 166 USPQ 204 (C.C.P.A. 1970))

Applicants point out that the comments recited above from Sambrook et al. establish a significant hurdle for the Examiner to establish a prima facie case that one of ordinary skill in the art would not ascertain with a reasonable degree of precision and particularity the invention defined by the subject claims. The Examiner has not presented any documentary evidence showing why one skilled in the art would not reasonably understand the scope of the "stringent hybridization condition" terminology. To the contrary, the Examiner states "the art recognizes a multitude of conditions which could be considered 'stringent'." Final Office Action, 04-04-06, page 7, paragraph 2. The Examiner thus admits one of ordinary skill in the art would understand the scope of the "stringent hybridization condition" terminology because for the art to recognize the existence of a multitude of satisfactory stringent conditions, they would necessarily need to understand the ultimate objective and thus the definition of stringent conditions. Sambrook's laboratory manual contains detailed descriptions of conditions for hybridization of oligonucleotide probes (p. 11.45), calculating or empirically determining melting temperatures (pp. 11.46, 11.55-11.57), and estimating effects of mismatched nucleotide pairs (p. 11.47). See Exhibit A of this Amendment After Final. Furthermore, based on the statement recited above from the April 2006 Final Action, the Examiner admits those of skill in the art at the time the priority application was filed knew "a multitude" of available stringent hybridization conditions. This further supports Applicants' contention that on of ordinary skill in the art would be able to determine if they fall within the scope of the claimed invention, and hence, would possess the skills and knowledge necessary to practice the invention as presented in the specification and defined in the subject claims. Applicants believe these documented facts establish "stringent hybridization conditions" were well understood in the art at the time the priority document was filed.

The sequence defined in the claims rejected in the present Office Action based on the "stringent hybridization terminology" establishes an isolated gene sequence species and defines the conditions necessary for identifying the scope of genus members. First, the disclosed sequence provides the necessary foundation for determining the conserved DNA, and more importantly non-conserved DNA, positions of the porcine leptin nucleotide genus when

compared to the known human and mouse sequences. One of ordinary skill in the art would understand the metes and bounds of the claimed invention when read in light of the specification.

Additionally, the priority application of the present application contains Example II and Example III that explicitly state stringent hybridization conditions. Those of ordinary skill in the art, based on the explanations provided in Applicants' prior Declaration Under 35 U.S.C. §132, would recognize the conditions explicitly recited in Examples II and/or III, for example, could be employed anytime stringent hybridization conditions are specified in a claim to satisfy that "stringent hybridization condition" claim language.

The Examiner alleges the claims defining "stringent hybridization conditions and present as of issuance of the present Office Action were indefinite "because the claims encompass a wide host of molecules depending on which conditions are intended by the terminology 'stringent hybridization conditions'". Contrary to this stated proposition, as noted above, the Examiner stated, "the art recognizes a multitude of conditions which could be used and considered stringent." It is also important to note that the claims specify the DNA binds to the identified sequence of the different claims under stringent hybridization conditions, rather than defining a method that employs stringent hybridization conditions. As the Examiner has implicitly noted, and the passages cited from Sambrook detail, one of ordinary skill in the art will recognize the objective of the claimed invention and would have the knowledge to choose from a variety of conditions to obtain the stringent conditions required to obtain the appropriate final result.

For the above stated reasons, Applicants believe the Examiner did not establish a prima facie case of indefiniteness for stringent hybridization condition in the present Office Action. The Examiner did not demonstrate the multitude of conditions known to one skilled in the art would not produce the appropriate results defined in the subject claims. Applicants respectfully suggest that an indefiniteness rejection based upon the "stringent hybridization condition" terminology is inappropriate in the face of Sambrook's articulation of the state of the art, without specific documentation that establishes one of ordinary skill in the art would not

been able to ascertain with a reasonable degree of precision and particularity the particular area set out and circumscribed by the subject claims.

Next, the Examiner continues to allege that use of the "substantially all" terminology in claims 16, 23-24, 26-28, 45, 56, 61, 67, 71, 75, 79, and 83-85 renders claims 16, 23-24, 26-28, 45, 56, 61, 67, 71, 75, 79, and 83-85 indefinite:

> Claims 16, 23-24, 26-28, 45, 56, 61, 67, 71, 75, 79, 83 and new claims 84-85 are directed to nucleic acid molecules (DNA, mRNA) which "hybridizes" to "substantially all" of the bases of a recited sequence. However, these claims are indefinite for the failure to indicate what is intended by the recitation "substantially all".
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> Applicant argues at pages 30-32 that "substantially all" is definite. Applicant's arguments have been carefully considered but have not been found to be persuasive. First, Applicant again refers to U.S. Pat. No. 6,756,484. Again, the Examiner will not comment on the prosecution of another application. This patent is not directed to nucleic acid molecules which hybridize to substantially all of the bases of a recited sequence. Therefore, it is not germane to the instant fact situation. Applicant's assertion of "differential treatment" is not supported by any facts of record.
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> The specification does not define "substantially all" and its use in conjunction with the indefinite "stringent hybridization conditions" clearly does not provide sufficient explanation of the metes and bounds of the claims. Applicant states that "the meaning of the term "substantially all" clearly means something less than "all," yet more than "half'. Applicant has provided no basis in the specification for this conclusion or definition. Applicant may mean 50%-100%, but someone in the art may view "substantially all" to be 80-100% while another researcher may view this to be 90-100%. Because the metes and bounds of what is being claimed is unclear, the claims are indefinite..

Despite the Examiner's allegations, Applicants disagrees that use of the "substantially all" terminology automatically renders claims 16, 23-24, 26-28, 45, 56, 61, 67, 71, 75, 79, and 83-85 that were in the present application when the present Office Action issued indefinite under the second paragraph of 35 U.S.C. §112. However, as noted above, Applicants have cancelled claims 16, 23-24, 26-28, 45, 56, 61, 67, 71, 75, 79, and 83-85 for reasons unrelated to the Examiner's indefiniteness rejection of claims 16, 23-24, 26-28, 45, 56, 61, 67, 71, 75, 79, and

83-85. Consequently, the Examiner's indefiniteness rejection of claims 16, 23-24, 26-28, 45, 56, 61, 67, 71, 75, 79, and 83-85 is moot.

Nonetheless, some clarifying comments about statements of the Examiner in support of the indefiniteness rejection are provided herein. The Examiner has not produced any evidence establishing that one of ordinary skill in the art would be unable to reasonably understand the scope of the "substantially all" claim language. Therefore, the Examiner has effectively done nothing more than question the definiteness of the "substantially all" claim language. This does not, however, establish that the subject claim language is in fact indefinite. The Examiner alleges that "Applicant may mean 50%-100%, but someone in the art may view 'substantially all' to be 80-100% while another researcher may view this to be 90-100%." Again, merely questioning the definiteness of the "substantially all" claim language with hypotheticals does not establish that the subject claim language is in fact indefinite.

Finally, the Examiner alleges that claims 84-85 suffer from an antecendent basis issue that renders claims 84-85 indefinite:

> New claims 84 and 85 recite the limitation "the porcine leptin polypeptide". There is insufficient antecedent basis for this limitation in the claim. The article "the" implies that there is a single porcine leptin polypeptide to which the claim is referring, but no such protein is referenced. The use of the article "a" in place of "the" would obviate this ground of rejection.

As noted above, Applicants have cancelled claims 84-85 for reasons unrelated to the Examiner's indefiniteness rejection of claims 84-85. Consequently, the Examiner's indefiniteness rejection of claims 84-85 is moot

Claim Rejections Under 35 U.S.C. §103(a) Based On The Friedman Patent

In the Office Action, the Examiner continues to reject claims 22-28, 42-62, 64-83 and now rejects claims 84-87 under 35 U.S.C. 103(a) as allegedly being unpatentable over U.S. Patent N. 6,309,853 to Friedman et al. (subsequently referred to as the "Friedman patent"). In support of this rejection, the Examiner now states:

The instant specification defines a functional derivative as

> Any "fragment", "variant", "analog" or "chemical derivative" of the porcine adipocyte polypeptide that retains at least a portion of the function of the porcine adipocyte polypeptide which permits its utility in accordance with the present invention. (page 9 of the specification).

The instant claims are directed to isolated nucleic acids which encode porcine leptin or a "functional derivative thereof' or "variant thereof'. The prior art of Friedman et al. (U.S. Pat. No. 6,309,853) disclose nucleic acids which encode human and mouse leptin, which would be considered functional derivatives and/or variants of the disclosed porcine leptin since they encode leptin molecules and would possess similar functional properties as those of the porcine leptin, absent evidence to the contrary. Friedman et al. teach that the leptin gene (or OB) could be isolated from domestic animals using the methods disclosed therein (see column 26, line 53 to column 27, line 49). Friedman et al. specifically mention swine as a domestic animal for which leptin would be useful (see column 48, lines 41-57). Friedman et al. do not specifically disclose an isolated nucleic acid encoding a porcine leptin polypeptide. However, it would have been obvious to use the nucleic acid of Friedman el al. encoding human or mouse leptin and hybridize it to a porcine cDNA library and isolate a nucleic acid molecule encoding porcine leptin because Friedman et al. teach methods for isolating leptin encoding nucleic acids and also teach that it would be beneficial to administer leptin to swine. It would also have been prima facie obvious to use the nucleic acid of Friedman et al. encoding human or mouse leptin and hybridize it to porcine genomic DNA to isolate the gene encoding porcine leptin because it would have been beneficial to more completely understand the gene structure of porcine leptin. It also would have been prima facie obvious to use the nucleic acid of Friedman et al. encoding human or mouse leptin and hybridize it to porcine mRNA to isolate the mRNA encoding porcine leptin for the benefit of understanding the nature of porcine leptin expression. Therefore, the invention as a whole would have been obvious at the time it was made, absent evidence to the contrary.

Applicant argues the rejection at pages 34-36 of the response. Applicant's arguments appear to be based on the premise that the porcine leptin of the instant application is functionally different from the human and mouse leptin of the prior art. However, the rejection is not one of anticipation, but rather that the human and mouse leptin of the prior art meet the limitation of being functional derivatives based on the disclosure of the instant specification at page 9. A ""functional derivative" refers to any "fragment", "variant", "analog", or

"chemical derivative" of the porcine adipocyte polypeptide that retains at least a portion of the function of the porcine adipocyte leptin" (see page 9 at lines 4-5). Therefore, Friedman et al. teach nucleic acid molecules which are "functional derivatives" and "derivatives" of the porcine leptin of the instant application and because they possess "at least a portion of the function of the porcine adipocyte leptin". Friedman et al. teach that the nucleic acid molecules encoding leptin could be used to isolate nucleic acid molecules encoding leptin from other species, specifically swine, contrary to Applicant's assertion that "the Friedman patent does not teach, suggest or disclose the invention of the above-identified application". The claims are broadly directed to isolated nucleic acids which encode porcine leptin - based on the known high degree of nucleic acid similarity of the leptin molecules across species (taught in Friedman), the known existence of a porcine leptin molecule (taught in Friedman), motivation to isolate nucleic acid molecules encoding porcine leptin (taught in Friedman) and known methods of isolation of nucleic acid molecules encoding leptin using one species as a probe (taught in Friedman), the invention as a whole would have been prima *facie* obvious in view of Friedman.

Applicant's arguments at pages 34-35 regarding specific activities of porcine leptin are noted, but do not avoid the rejection of record. The claims do not require these specific activities and the specification only requires "at least a portion of the function of the porcine adipocyte leptin". This function would include any function, such as binding to a leptin receptor, antigenicity, etc. Therefore, Applicant's arguments are not persuasive.

Applicant argues that "the Examiner switched horses and basically alleged Applicants could only consider functional properties disclosed for porcine leptin in the present application. This is an erroneous and overly restrictive view by the examiner." Applicant's arguments have been considered, but are not persuasive. The claims do not require the isolated molecule to encode a porcine leptin with any particular biological activity. If one of ordinary skill in the art used the polynucleotides of Friedman et al. to hybridize to porcine polynucleotides using the methods taught in Friedman et al., there is more than a reasonable expectation of success in isolating a porcine version of leptin, absent evidence to the contrary.

Applicant argues at the bottom of page 35-page 36 that the Examiner merely makes conclusions and does not properly reject the claims under 103. In response to applicant's argument that the examiner's conclusion of obviousness is based upon improper hindsight reasoning, it must be recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But so long as it takes into account only knowledge which was within

> the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. See *In re McLaughlin*, 443 F.2d 1392, 170 USPQ 209 (CCPA 1971). Furthermore, the rejection was based on the disclosure of Friedman, the success Friedman had in isolating a different species of leptin while using another species as a probe, the disclosure that leptin existed in pigs, and the specific statement of motivation in Friedman to isolate the molecules from other species, including pigs. Applicant has not provided any evidence on the record that one of ordinary skill in the art could not follow the teachings and guidance in Friedman et al. to isolate nucleic acids encoding leptin in pigs. The fact that the encoded protein has some very specific biological properties in the pig is interesting, but not persuasive for the reasons given above and does not avoid the rejection of record.

Despite the Examiner's allegations, the Friedman patent does not teach, suggest, disclose, or make obvious the invention of the above-identified application, as defined in claims 22-28, 42-62, and 64-87. However, as noted above, Applicants have cancelled claims 22-28, 42-62, and 64-87 for reasons unrelated to the Examiner's rejection of claims 22-28, 42-62, and 64-87 under 35 U.S.C. 103(a) based on the Friedman patent. Consequently, the Examiner's rejection of claims 22-28, 42-62, and 64-87 under 35 U.S.C. 103(a) based on the Friedman patent is moot.

Nonetheless, some clarifying comments about statements of the Examiner in support of the rejection under 35 U.S.C. 103(a) based on the Friedman patent are provided herein. Consistent with the Examiner's observation, the Friedman patent does disclose murine and human leptin DNA sequences and polypeptides. Also, consistent with the Examiner's observation, the Friedman patent does not disclose any porcine leptin DNA (or mRNA) molecules or polypeptides. Furthermore, consistent with the Examiner's observation, the Friedman patent does not disclose any functional derivative or variant DNA (or mRNA) molecules that encode for porcine leptin polypeptide.

For example, when recombinant porcine leptin protein is administered by intracerebroventricular (ICV) injection to crossbred prepuberal gilts (normal female pigs), the crossbred prepuberal gilts exhibit increased growth hormone secretion after the leptin administration. On the other hand, when recombinant human leptin protein is administered by ICV injection to normal male rats, the normal male rats do not exhibit increased growth hormone

secretion after the leptin administration. Based on the documented differences in growth hormone secretion after ICV administration to normal fed pigs (per the previously cited Barb publication) versus normal fed rats (per the previously cited Carro publication), it is evident that recombinant porcine leptin protein surprisingly functions very differently from recombinant human leptin protein upon administration to mammals.

As another example, when recombinant porcine leptin is administered by intracerebroventricular (ICV) injection to crossbred prepuberal gilts (normal female pigs), the recombinant porcine leptin administration fails to change thyroxine (T_4) secretion. On the other hand, when recombinant mouse leptin is administered by ICV injection to normal ad libitum fed male rats, the normal ad libitum fed male rats exhibit significantly decreased thyroxine (T_4) levels in the blood after the recombinant mouse leptin administration. Since recombinant porcine leptin administration fails to change thyroxine (T_4) secretion in pigs, while recombinant murine leptin administration significantly decreased thyroxine (T_4) levels in the blood of male rats, the effects of porcine leptin administration and murine leptin administration differ dramatically, and it is evident the porcine leptin functions very differently from the murine leptin upon administration to mammals.

The Examiner suggests the Friedman patent "discloses nucleic acids which encode human and mouse leptin, which would be considered functional derivatives and/or variants of the disclosed porcine leptin since they encode leptin molecules and would possess similar functional properties as those of the porcine leptin, absent evidence to the contrary." Applicants have provided such evidence demonstrating that Friedman nucleic acids which encode human and mouse leptin do not possess similar functional properties as the properties of porcine leptin. This evidence is summarized in the previous two paragraphs of this Amendment. Applicants' factual evidence illustrates the human leptin disclosed in the Friedman patent does not, despite the Examiner's contentions to the contrary, necessarily, or actually, possess functional properties similar to the functional properties of the porcine leptin disclosed in the above-identified application. Nonetheless, in response to this presentation of differing properties, the Examiner switched horses and basically alleged Applicants could only consider functional properties disclosed for porcine leptin in the present application.

This is an erroneous and overly restrictive view by the examiner. The evidence Applicants have already presented demonstrates functional differences between the Freidman leptin and porcine leptin. The fact that those differences exist with some functional attributes raises serious doubts about the correspondence in other functional attributes, such as those disclosed in the present application, despite the Examiner's attempt to avoid this evidence. Consequently, it is clear Applicant has rebutted the Examiner's prima facie case of obviousness and it is the Examiner's obligation to now withdraw the present rejection or present contrary evidence demonstrating correspondence in other functional attributes between the Freidman leptin and porcine leptin.

Furthermore, the Examiner relies on purely conclusory argument in support of the alleged obviousness of creating the porcine invention of the present application based on the disclosure of the Friedman patent. Specifically, the Examiner relies on blue sky "knowledge base" enhancements the Examiner pulls out of thin air as the alleged grounds for attempting to hybridize human or murine leptin to porcine DNA:

> It would also have been prima facie obvious to use the nucleic acid of Friedman at al. encoding human or mouse leptin and hybridize it to porcine genomic DNA to isolate the gene encoding porcine leptin because it would have been beneficial to more completely understand the gene structure of porcine leptin. It also would have bean prima facie obvious to use the nucleic acid of Friedman at al. encoding human or mouse leptin and hybridize it to porcine mRNA to isolate the mRNA encoding porcine leptin for the benefit of understanding the nature of porcine leptin expression.

Thus, the "suggestion" comes from the mind of the Examiner, rather than as any real and tangible suggestion provided by the art. Such suggestions developed in the absence of corroboration by the art are suspect. Such suggestions developed in the absence of corroboration by the art are particularly useful when attempting to support an obviousness allegation that is in fact based on hindsight reconstruction. Generalized "knowledge base" enhancement necessarily must fail as a basis for obviousness since success of such a basis, when aimed in any direction about the art, would bring invention to a halt and to the knees of the Friedman patent. This is true, because one could always dream up some nice potential use for the enhancing the general knowledge base, once it was known what target one is trying to bring to a halt.

Continuing, based on the factual results noted above and despite the Examiner's contentions to the contrary, the Examiner's speculative suggested hybridization of the nucleic acid

of the Friedman patent that encodes murine leptin to a porcine DNA library and subsequent isolation of a nucleic acid molecule encoding porcine leptin is not suggested for yet another reason. Specifically, based on the known differences between functional attributes of porcine leptin and murine leptin, one of ordinary skill in the art would not expect the functional characteristics of the murine leptin disclosed in the Friedman patent would be helpful for confirming isolation of a nucleic acid molecule encoding for porcine leptin, as claimed in the above-identified application. This demonstrated lack of correspondence between functional properties of murine leptin versus porcine leptin would instead suggest that such a project would not be advisable. Analogous reasoning applies with regard to human leptin, since the differences in functional characteristics of the human leptin disclosed in the Friedman patent versus the functional characteristics of the porcine leptin of the present invention would not support confirmation of isolation of a nucleic acid molecule encoding for porcine leptin, as claimed in the above-identified application.

Finally, the US 08/692,922 priority application presents the nucleotide sequences for human and mouse leptin polypeptides (as depicted in Figure 4 a corresponding sequence of SEQ ID NO:1, SEQ ID NO:6, and SEQ ID NO: 5) that teach the art away from the actual porcine leptin nucleotide sequence of the current invention in three critical respects related to the art's ability to make use of stringent hybridization conditions: (1) the porcine sequence has 33 positions that are not conserved with either the human or mouse gene sequences, (2) the porcine nucleotide sequence has 51 additional positions that are not conserved with the mouse sequence, and (3) further the porcine nucleotide sequence has an additional 28 positions that are not conserved with the human sequence. (as depicted in Figure 4 a corresponding sequence of SEQ ID NO:1, SEQ ID NO:6, and SEQ ID NO: 5) In total, the porcine nucleotide sequence of the current invention contains one hundred and eleven distinctive features that were not taught nor suggested by the prior art sequences. For these reasons the porcine nucleotide sequence of the current invention is both novel and non-obvious with respect to the application of this information to DNA-DNA hybridization.

The 33 positions of the porcine sequence not conserved with either the mouse and human sequences are at positions 9, 31, 43, 55, 59, 64, 74, 75, 78, 111, 129, 144, 151, 190, 202, 205, 206, 235, 241, 242, 295, 339, 362, 264, 265, 266, 370, 372, 397, 415, 427, 458, and 499. (as

depicted in Figure 4 a corresponding sequence of SEQ ID NO:1, SEQ ID NO:6, and SEQ ID NO: 5) These positions in the porcine sequence are important to the current invention because they provide unique identifiers for determining the porcine leptin nucleotide genus sequence. This genus was not and could not be identified from the prior art. More specifically, the prior art taught away from these porcine leptin nucleotide genus configurations. The prior art specifically teaches conserved nucleotides between human and mouse at 22 of the 23 identified non-conserved positions. (as depicted in Figure 4 a corresponding sequence of SEQ ID NO:1, SEQ ID NO:6, and SEQ ID NO: 5) These features support the proposition the porcine leptin nucleotide genus of sequences are novel and non-obvious over the available human and mouse sequences.

The current inventive porcine sequence has an additional nucleotide at position 31 not found in human or mouse, and the porcine sequence is missing the corresponding nucleotides at positions 364, 365, and 366 found in both human and mouse sequences. (as depicted in Figure 4 a corresponding sequence of SEQ ID NO:1, SEQ ID NO:6, and SEQ ID NO: 5) Again, these unique features of the porcine leptin nucleotide genus were not and could not have been observed nor predicted from the prior art teachings. Thus, these features support the proposition the porcine leptin nucleotide genus of sequences are novel and non-obvious over the prior art.

The isolated porcine leptin nucleotide sequence is not conserved, contrary to the expected results as based upon the prior art teachings of human and mouse leptin nucleotide sequences, at sequence positions 4, 5, 6, 10, 13, 21, 22, 24, 28, 41, 49, 51, 52, 64, 70, 76, 85, 112, 151, 154, 158, 163, 168, 168, 175, 178, 199, 208, 213, 214, 217, 235, 247, 254, 265, 266, 275, 276, 280, 284, 286, 289, 293, 298, 310, 316, 319, 322, 325, 328, 329, 343, 353, 354, 356, 361, 362, 363, 365, 377, 381, 382, 383, 384, 385, 388, 396, 397, 412, 416, 417, 439, 440, 457, 472, 475, 476, 477, 478, 482, 487, 488, 490, 498, and 499 (as depicted in Figure 4 a corresponding sequence of SEQ ID NO:1, SEQ ID NO:6, and SEQ ID NO: 5.)

The current invention shows 50 nucleotides in the porcine sequence, of the total 85 non-conserved positions in human and mouse leptin, are conserved with only the human leptin sequence; these positions are located at 4, 10, 21, 24, 41, 52, 70, 154, 158, 163, 175, 178, 199, 208, 247, 254, 265, 275, 276, 284, 286, 289, 293, 310, 316, 322, 328, 353, 356, 361, 363, 377, 381, 382, 383, 384, 385, 396, 439, 440, 457, 472, 475, 477, 478, 482, 487, 488, 490, and 498 (as

depicted in Figure 4 a corresponding sequence of SEQ ID NO:1, SEQ ID NO:6, and SEQ ID NO: 5.) The prior teaches the art to expect 85 out of a total 505 base pairs will show variation. The current invention shows an even greater variation, 111 nucleotide variations out of a total 505 base pairs, when compared to the combined human and mouse prior art sequences. This is an unexpected increase of 22 percent in variability. Thus, based upon the original priority application as filed, US 08/692,922, one of ordinary skill in the art would understand how to take advantage of the sequence variances of the inventive disclosure to identify DNA sequences through the use of hybridization conditions to demonstrate the full scope the porcine leptin nucleotide genus encompasses.

New Claims Added by Applicants

Applicants have added new claims 88-122. New claims 88-122 do not add any new matter to the above-identified application. Support for new claims 88-122 is believed to exist throughout the above-identified application. Applicants respectfully request consideration and allowance of new claims 88-122.

CONCLUSION

New claims 88-122 are believed allowable. Therefore, consideration and allowance of new claims 88-122 is respectfully requested. The Examiner is invited to contact Applicants' below-named attorney, Philip F. Fox, to facilitate allowance of the above-identified application.

Respectfully submitted,

Date: October 4, 2006

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OF

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IDENTIFICATION OF cDNA CLONES OF INTEREST

Methods of Screening

There are three methods to screen cDNA libraries for clones of interest:

- Nucleic acid hybridization
- Immunological detection of specific antigens
- Sib selection either by hybrid selection and translation of mRNA or by production of biologically active molecules

Most cloning projects today are aimed at isolating cDNAs corresponding to rare mRNAs and therefore require screening of large numbers of recombinant clones. This can be carried out effectively with only two types of reagents: antibodies and nucleic acid probes. In those rare instances when both types of reagents are available, nucleic acid probes are preferred because they can be used under a variety of different stringencies that minimize the chance of undesirable cross-reactions. Furthermore, nucleic acid probes will detect all clones that contain cDNA sequences, whereas antibodies will react only with a subset of these clones (in some cases one in six at best) in which the cDNA has been inserted into the vector in the correct reading frame and orientation. cDNA libraries that are to be screened by antibodies therefore need to be larger (by a factor of at least 6) than those that are to be screened by nucleic acid probes. Consequently, when using antibody probes to search for a cDNA clone corresponding to a mammalian mRNA present at the level of 1 molecule/cell or less, it is desirable to construct cDNA expression libraries that contain in excess of 10^7 members. This is not easy, especially when the amounts of mRNA are limited. Furthermore, screening a library of this size is expensive and laborious, and it becomes worthwhile to explore methods to enrich the mRNA (or cDNA derived from it) for the sequences of interest (see pages 8.6–8.10).

NUCLEIC ACID HYBRIDIZATION

This is the most commonly used and reliable method of screening cDNA libraries for clones of interest. None of the other methods displays such an abundance of attractive features. Screening by nucleic acid hybridization allows extremely large numbers of clones to be analyzed simultaneously and rapidly, does not require that the cDNA clones be full-length, and does not require that an antigenically or biologically active product be synthesized in the host cell. Furthermore, as a result of more than 20 years of work, the theoretical basis of nucleic acid hybridization is well-understood. This has led to the development of a large number of different techniques that can accommodate nucleic acid probes of very different lengths and specificities. Details of the methods for the preparation and use of these probes are presented in Chapters 10 and 11.

Homologous probes

Homologous probes contain at least part of the exact nucleic acid sequence of the desired cDNA clone. They are used in a variety of circumstances, for

Conditions for Hybridization of Oligonucleotide Probes

When using oligonucleotides as probes, the aim is to find conditions that are stringent enough to guarantee specificity and sufficiently flexible to allow formation of stable hybrids at an acceptable rate. For DNA molecules more than 200 nucleotides in length, hybridization is usually carried out at 15–25°C below the calculated melting temperature (T_m) of a perfect hybrid. However, as the length of the probe is decreased, the T_m is lowered to the point where it is often impractical to carry out hybridization at T_m – 25°C. Typically, therefore, hybridization with synthetic oligonucleotides is carried out under conditions that are only 5–10°C below the T_m. Although such stringent conditions reduce the number of mismatched clones that are detected with short oligonucleotide probes, they have the less desirable consequence of reducing the rate at which perfect hybrids form.

Hybrids formed between DNA molecules more than 200 nucleotides in length are completely stable for all practical purposes. The chances that such a long stretch of double helix will unwind at temperatures 15–25°C below the T_m are extremely small. However, hybrids (even perfect hybrids) formed between short oligonucleotides and their target sequences at 5–10°C below the T_m are far easier to unwind, and hybridization reactions of this type can be regarded as reversible. This has important practical consequences. Whereas hybrids formed between longer DNA molecules are essentially stable under the conditions used for posthybridization washing, hybrids (even perfect hybrids) involving short oligonucleotides are not. Posthybridization washing of such hybrids must therefore be carried out rapidly so that the probe does not dissociate from its target sequence. For this reason, hybridizations with short oligonucleotides should be carried out under stringent conditions (5–10°C below the T_m) using high concentrations (0.1–1.0 pmole/ml) of probe. When only one or a small number of oligonucleotides (<8) are used as probes, the annealing reaction rapidly reaches equilibrium, and hybridization should therefore be terminated after 3 or 4 hours. More complex mixtures, in which the concentration of each oligonucleotide is comparatively low, require hybridization to be carried out for proportionately longer periods. For example, mixtures of 32 or more oligonucleotides should be hybridized for 1–2 days. Posthybridization washing should be carried out for brief periods of time, initially under conditions of low stringency and then under conditions of stringency equal to those used for hybridization.

CALCULATING MELTING TEMPERATURES FOR PERFECTLY MATCHED HYBRIDS BETWEEN OLIGONUCLEOTIDES AND THEIR TARGET SEQUENCES

When using single oligonucleotides that match the target sequence perfectly, hybridization conditions can easily be derived from the calculated T_m of the hybrid. For oligonucleotides shorter than 18 nucleotides, the T_m of the hybrid can be estimated by multiplying the number of A + T residues in the hybrid by 2°C and the number of G + C residues by 4°C and adding the two numbers (Itakura et al. 1984). However, this method overestimates the T_m of hybrids involving longer oligonucleotides.

A different approach has been taken by E. Fritsch (unpubl.), who found that the equation originally used to calculate the relationship between G + C content, ionic strength of the hybridization solution, and the T_m of long DNA molecules (Bolton and McCarthy 1962):

$$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%\,G + C) - (600/N),$$

where N = chain length, predicts reasonably well the T_m for oligonucleotides as long as 60–70 nucleotides and as short as 14 nucleotides.

ESTIMATING THE EFFECTS OF MISMATCHES

Perhaps surprisingly, the classic formula (Bonner et al. 1973) to calculate the effect of mismatches on the stability of long DNA hybrids holds reasonably well for hybrids involving short oligonucleotides: For every 1% of mismatching of bases in a double-stranded DNA, there is a reduction of T_m by 1–1.5°C. However, the precise effect of mismatches depends on the G + C content of the oligonucleotide and, even more critically, on the distribution of mismatched bases in the double-stranded DNA. Mismatches in the middle of the oligonucleotide are far more deleterious than mismatches at the ends. Therefore, the method of estimation given above should only be used as a rough guide until a systematic study of all types of mismatches in a variety of contexts leads to more precise methods of estimation. If appropriate target DNA has been cloned, the effect of mismatches on T_m can be determined empirically (see pages 11.55–11.57).

EMPIRICAL DETERMINATION OF MELTING TEMPERATURE

The melting temperature (T_m) of an oligonucleotide hybridized to a target sequence can be determined by the procedure described below. The protocol actually measures the temperature at which dissociation of the double-stranded DNA becomes irreversible (T_i) in nonequilibrium conditions that do not favor rehybridization of the released probe to the target. The optimal temperature for hybridization is then determined on the basis of this value. The procedure requires a cloned target sequence that is complementary (perfectly or imperfectly, depending on the experiment) to the oligonucleotide probe. In most cases, a target sequence is not available from "natural" sources and must be synthesized chemically. The best synthetic target sequences consist of two oligonucleotides that are partially complementary. After annealing, these oligonucleotides form a double-stranded region that contains the target sequence. The sequences of the protruding ends are designed to allow the target DNA to be cloned easily in bacteriophage M13 vectors. Single-stranded DNA of the appropriate orientation prepared from the resulting clones (see Chapter 4) can be used in hybridization experiments as described below. It can also be used as a template for dideoxy-mediated chain-termination sequencing (see Chapter 13) if it is necessary to check that the sequence of the target DNA is correct.

1. Label 1–10 pmoles of the oligonucleotide to be used as a probe by phosphorylation (see pages 11.31–11.32), and remove excess unincorporated [γ-^{32}P]ATP by one of the methods described on pages 11.33–11.39.

2. Using a paper punch, prepare four small circles (diameter 3–4 mm) of a solid support (nitrocellulose filter or nylon membrane) used for hybridization. Arrange the small circles on a piece of Parafilm. Mark two of the filters with a soft-lead pencil.

3. Apply approximately 100 ng of target single-stranded DNA in a volume of 1–3 μl of 2× SSC to each of the marked filters. Apply an equal amount of vector DNA to the unmarked filters. After the fluid has dried, use blunt-ended forceps (e.g., Millipore forceps) to remove the two sets of filters from the Parafilm, and place them between sheets of Whatman 3MM paper. Fix the DNAs to the filters by baking for 1–2 hours at 80°C in a vacuum oven.

   If the target DNA has been cloned into a plasmid, linearize the vector by digestion with a restriction enzyme that does not cleave within the target sequences. Purify the resulting double-stranded DNA by extraction with phenol:chloroform and precipitation with ethanol. Dissolve the DNA in 2× SSC at a concentration of 500 ng/μl. Apply the solution of DNA to the filters prepared as described above, and then, using blunt-ended forceps, transfer the filters to a sheet of 3MM paper saturated with denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 5–10 minutes. Move the filters to a fresh sheet of 3MM paper saturated with neutralizing solution (0.5 M Tris·Cl [pH 7.4], 1.5 M NaCl) for 10 minutes. Transfer the filters to a dry sheet of 3MM paper, and leave them at room temperature until all of the fluid has evaporated. Bake the filters as described above.

   Overbaking can cause the filters to become brittle. In addition, filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

4. Using blunt-ended forceps, transfer all of the filters to a polyethylene tube that contains 2 ml of oligonucleotide prehybridization solution. Seal the tube and incubate, with occasional shaking, at a temperature estimated to be $T_m - 25°C$ for the solvent being used (see Note i). After 2 hours, add radiolabeled oligonucleotide to the prehybridization solution. The final concentration of oligonucleotide should be approximately 1 pmole/ml. Continue incubation at $T_m - 25°C$ for a further 2–4 hours, with occasional shaking.

Oligonucleotide prehybridization solution

6× SSC (or 6× SSPE)
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 8.0)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA (see Appendix B)
0.1% nonfat dried milk

5. Remove the filters from the hybridization solution, and immediately immerse them in 2× SSC at room temperature. Agitate the fluid continuously. Replace the fluid every 5 minutes until the amount of radioactivity on the filters remains constant (as measured with a hand-held minimonitor).

6. Adjust the temperature of a circulating water bath to $T_m - 25°C$. Dispense 5 ml of 2× SSC into each of 20 glass test tubes (17 mm × 100 mm). Monitor the temperature of the fluid in one of the tubes with a thermometer. Incubate the tubes in the water bath until the temperature of the 2× SSC is $T_m - 25°C$. The 2× SSC in each of these tubes will be used separately for each temperature increase (see steps 7–10).

7. Transfer the filters individually to four empty glass tubes, separating the filters containing the vector and target DNAs, and add 1 ml of 2× SSC (from one of the tubes prepared in step 6 and prewarmed to $T_m - 25°C$). Place the tubes in the water bath for 5 minutes.

8. Remove the tubes from the bath, transfer the liquid to scintillation vials, and wash the tubes and filters with 1 ml of 2× SSC at room temperature. Add the wash solutions to the appropriate scintillation vials.

9. Increase the temperature of the water bath by 3°C, and wait for the temperature of the 2× SSC in the tubes prepared in step 6 to equilibrate.

10. Add 1 ml of 2× SSC at the higher temperature to each of the four tubes containing the filters. Place the tubes in the water bath for 5 minutes.

11. Repeat steps 8, 9, and 10 at successively higher temperatures until a temperature of $T_m + 30°C$ is achieved.

12. Place the filters in separate glass tubes (17 mm × 100 mm) containing 1 ml of 2 × SSC, and heat them to boiling for 5 minutes to remove any remaining radioactivity. Cool the solutions in ice, and transfer them to scintillation vials. Wash the filters and tubes used for boiling with 1 ml of 2 × SSC, and add the washing solutions to the appropriate scintillation vials.

13. Use a scintillation counter to measure the radioactivity (by Cerenkov counting, see Appendix E) in all of the vials. Calculate the proportion of the total radioactivity that has eluted at each temperature (i.e., the sum of radioactivity eluted at all temperatures between $T_m - 25°C$ and the temperature at which a given sample was taken divided by the total radioactivity eluted from the filters at all temperatures up to and including 100°C).

    If the experiment has worked well, very little radioactivity should be associated with the filters containing vector DNA alone. Furthermore, this radioactivity should be completely released from the filters at temperatures much lower than the estimated T_m. On the other hand, considerable radioactivity should be associated with the filters containing the target DNA; the elution of this radioactivity should show a sharp temperature dependence. Very little radioactivity should be released from the filters until a critical temperature is reached, and then approximately 90% of the radioactivity should be released during the succeeding 6–9°C rise in temperature. The temperature at which 50% of the radioactivity has eluted from the filters containing the target sequences is defined as the T_i of the hybrid between the probe and its target sequence.

Notes

i. Although the above protocol calls for the use of sodium salts in the solvent used for hybridization, other solutes such as tetramethylammonium chloride or tetraethylammonium chloride can be substituted if desired to determine the T_i in these solvents.

ii. This method can easily be adapted to study the behavior of hybrids formed between probes and target sequences that do not match each other perfectly (Jacobs et al. 1988).

iii. Before synthesizing the probe, check for potential homology and/or complementarity between its sequence and the sequence of the vector used to propagate the target. Most of the commercially available programs to analyze DNA can be used to search commonly used vectors for sequences that match the sequence of the probe closely enough to cause problems during hybridization.